Identification and purification of a liver microsomal glucose 6-phosphatase

Ann BURCHELL and Brian BURCHELL
Department of Biochemistry, Medical Sciences Institute, University of Dundee, Dundee DD1 4HN, Scotland, U.K.

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1. Hepatic glucose 6-phosphatase activity was purified 65-fold in good yield over that in cholate-solubilized microsomal fractions. 2. This preparation still contained five major polypeptides and numerous minor contaminants. 3. The smallest of the five major polypeptides (M, approx. 18 500) could be purified from heat-treated microsomal fractions. 4. Antiserum raised against the heat-stable protein doublet was used to immunoprecipitate specifically glucose 6-phosphatase activity from cholate-solubilized microsomal fractions. 5. This work indicates that hepatic microsomal glucose 6-phosphatase appears to be one or both of the low-molecular-weight heat-stable polypeptides.

Glucose 6-phosphate occupies a central crossroad position in hepatic carbohydrate metabolism (Nordlie, 1971). Under normal conditions 55% of the glucose 6-phosphate appears to be utilized by glucose 6-phosphatase (EC 3.1.3.9) (Ashmore et al., 1957). This phosphohydrolase appears to play a key role in the strict control of blood glucose concentrations (Cori & Cori, 1952).

The extreme lability of the 'solubilized' glucose 6-phosphatase (Beaufay & De Duve, 1954; Garland et al., 1974) is undoubtedly the major reason for repeated failure to purify the enzyme. However, we shall not be able to study the molecular basis of the enzyme deficiency in Von Gierke's disease (Hers, 1964) or understand the mechanisms involved in the 'mandatory' regulation of this enzyme activity (Stadtman, 1966), unless we obtain purified preparations of this enzyme.

Previously we have demonstrated that solubilized and partially purified glucose 6-phosphatase activity could be stabilized by the presence of 20 mM-NaF (Burchell & Burchell, 1980). Thus we have been able to obtain a highly purified preparation of glucose 6-phosphatase by conventional methods, as described below. We also show that the glucose 6-phosphatase protein can be isolated from heat-treated microsomal fractions and used as a source of antigen for the identification and purification of active glucose 6-phosphatase by immunochemical methods.

Materials and methods

Glucose 6-phosphatase and sodium cholate were from Sigma (London). Egg phosphatidylcholine was purchased from Lipid Products, South Nutfield, Surrey, U.K. Polyethylene glycol 6000 and NaF were from BDH, Poole, Dorset, U.K.

Enzyme assays

Glucose 6-phosphatase activity was assayed at 37°C in a 0.1 ml reaction mixture as described by Bickerstaff & Burchell (1980). One unit of activity represents 1.0 μmol of P<sub>i</sub> released/min. Non-specific hydrolysis of glucose 6-phosphate was assayed as described by Burchell & Burchell (1980). It averaged <3% of the total glucose 6-phosphatase activity in microsomal fractions. UDP-glucuronyltransferase activity was measured with 4-nitrophenol as substrate by the method of Winsnes (1969). Glutathione S-transferase activity was measured with 1-chloro-2,4-dinitrobenzene as described by Habig et al. (1974). Protein concentrations were determined by the method of Bradford (1976). Phosphatidylcholine liposomes were prepared as described by Cater et al. (1975) and added to enzyme fractions as indicated in the text.

Partial purification of glucose 6-phosphatase

Three fresh livers (total 300 g) from adult female New Zealand White rabbits were washed and then homogenized in ice-cold 20 mM-NaF/0.25 M-sucrose and microsomal pellets isolated as described by Burchell (1977). The microsomal pellets were
resuspended in buffer A [1% (w/v) sodium cholate, 20% (v/v) glycerol, 20 mM-NaF, in 0.1 M-tris/HCl, pH 7.0] by gentle homogenization by hand. After centrifugation for 60 min at 105,000 g at 4°C, the supernatants were collected and adjusted to 20 mg of protein/ml with buffer A (fraction 1). Polyethylene glycol (25%, w/v) was mixed with fraction 1 at 0°C until the final concentration was 3%. This mixture was stored at −20°C overnight. After thawing in ice, the mixture was diluted 5-fold with ice-cold buffer B [20% (v/v) glycerol, 1 mM-EDTA, 0.5 mM-dithiothreitol in 0.1 M-tris/HCl, pH 7.0]. After centrifugation at 105,000 g for 30 min at 4°C, the pellets were collected and resuspended in a buffer A/buffer B mixture (3:1, v/v) to a protein concentration of 4 mg/ml (fraction 2). After a further centrifugation at 105,000 g for 60 min at 4°C the pellets were resuspended in buffer B (fraction 3). An equal volume of buffer B plus KCl (2 M) was added to fraction 3, and the mixture was applied to a phenyl-Sepharose column (10.5 cm × 2.5 cm; Pharmacia, Uppsala, Sweden) previously equilibrated with 1 M-KCl in buffer B. The column was eluted with 1 M-KCl in buffer B and the eluate collected (fraction 4). Fraction 4 was centrifuged at 105,000 g for 60 min at 4°C. The pellet was redissolved in buffer A (fraction 5). An equal volume of buffer A plus 2 M-KCl was added to fraction 5 and the mixture centrifuged at 105,000 g for 60 min at 4°C. The final pellet was resuspended in buffer B (fraction 6).

Purification of the heat-stable protein

Microsomal pellets from 150 g of fresh or frozen rabbit liver were prepared as previously described. Microsomal pellets were resuspended in 20 mM-NaF/0.25 M-sucrose (20 mg of protein/ml). Samples (1 ml) of the suspension were heated in Eppendorf tubes at 100°C for 2 min. After heating, the suspensions were centrifuged at 8500 g for 2 min. The supernatants were collected and pooled and further centrifuged at 105,000 g for 90 min. The purified heat-stable protein in the pellets were resuspended in 20 mM-NaF/0.25 M-sucrose.

Preparation of antiserum to the heat-stable protein

Antisera against the heat-stable protein were raised in White Leghorn hens by the method described by Cohen et al. (1976). The IgG fraction was purified as previously described (Cohen et al., 1976).

Immunoprecipitation of glucose 6-phosphatase activity

Samples of cholate-soluble enzyme preparation (5 μl of fraction 1) were incubated with 45 μl of buffer A and 50 μl of antiserum for 60 min in ice. The final concentration of cholate in the reaction mixture was 0.5% (w/v). After incubation the reaction mixture was centrifuged at 8000 g for 2 min. The resulting immunoprecipitates were washed five times by resuspension in buffer A and re-centrifugation. Final immunoprecipitates were then resuspended in 100 μl of buffer A.

Immunoprecipitations of glucose 6-phosphatase by purified IgG fractions were performed by the same method, except that the reaction mixture contained 90 μl of IgG, 5 μl of cholate-soluble enzyme and 5 μl of buffer A. Thus the final concentration of cholate in the incubation mixture was lowered to 0.1% (w/v) (see the Discussion section).

Phosphatidycholine liposomes (4 mg/ml) were mixed with immunoprecipitates for 10 min at 20°C before enzyme assay.

Gel electrophoresis

Disc gel electrophoresis was performed in 7.5% polyacrylamide gels in the presence of 0.1% SDS at 20°C, pH 7.2, and a constant 60 V (Weber & Osborn, 1965). Slab gradient (4–23%) polyacrylamide-gel electrophoresis was performed at 20°C in the presence of 0.1% SDS as described by Laemmli (1970). The molecular weight of the purified heat-stable protein was determined by comparison with the mobility of cytochrome c (12,500), whale myoglobin (17,200), carbonic anhydrase (29,500) and ovalbumin (43,000).

Results

Purification of rabbit liver glucose 6-phosphatase

Glucose 6-phosphatase was solubilized from rabbit liver microsomal pellets (Bickerstaff & Burchell, 1980). This procedure released over 95% of the activity into the cholate supernatant (fraction 1). This glucose 6-phosphatase activity was then purified a further 65-fold, as shown in Table 1. Phosphatidycholine liposomes (4 mg/ml) were added back to fractions 4, 5 and 6 before assay. The best final specific activity obtained was 17.6 units/mg of protein in an approx. 30% yield. After many attempts using ion-exchange materials, gel filtration, hydroxyapatite, hydrophobic materials and possible affinity ligands we were unable to improve on this purification, owing to the insolubility of the salt pellets.

Examination of fraction 6 by SDS/polyacrylamide-disc-gel electrophoresis revealed the existence of five major protein-staining bands, but also numerous minor contaminants (Fig. 1). However, from the extent of purification of glucose 6-phosphatase activity, we believed that the enzyme must be one of the major protein components of this preparation. Thus we tried to prepare antisera to each of these five proteins.
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Table 1. Purification of rabbit liver glucose 6-phosphatase

Data presented are from a single purification experiment. Numbers in parentheses are ranges of values obtained during at least 30 separate preparations.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Specific activity (μmol/min per mg of protein)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cholate supernatant</td>
<td>110</td>
<td>2910</td>
<td>0.27 (0.19–0.37)</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>2. Polyethylene glycol precipitate</td>
<td>260</td>
<td>1015</td>
<td>0.52 (0.44–1.9)</td>
<td>68</td>
<td>2</td>
</tr>
<tr>
<td>3. Cholate precipitate</td>
<td>28</td>
<td>239</td>
<td>1.7 (0.86–2.7)</td>
<td>51</td>
<td>6.3</td>
</tr>
<tr>
<td>4. Phenyl-Sepharose eluate</td>
<td>888</td>
<td>88</td>
<td>4.3 (2.3–4.3)</td>
<td>47</td>
<td>15.9</td>
</tr>
<tr>
<td>5. Eluate precipitate</td>
<td>20</td>
<td>55</td>
<td>4.7 (2.3–4.)</td>
<td>33</td>
<td>17.4</td>
</tr>
<tr>
<td>6. KCl pellet</td>
<td>9</td>
<td>14</td>
<td>17.6 (6.3–17.6)</td>
<td>31</td>
<td>65.2 (17–93)</td>
</tr>
</tbody>
</table>

Fig. 1. SDS/polyacrylamide-gel electrophoresis of purified glucose 6-phosphatase

Disc electrophoresis was performed in 7.5% cylindrical (7.0 cm x 0.6 cm) gels in the presence of 0.1% SDS (see the Materials and methods section). Key: Fr.6, fraction-6 KCl pellet (see Table 1); HSP, purified heat-stable proteins. Gels were stained with Coomassie Brilliant Blue for 60 min and destained with acetic acid/methanol/water (7:5:43, by vol). The direction of migration is from the top to the bottom (df, dye front).

Purification of liver microsomal heat-stable protein

We heat-treated rabbit liver microsomal suspensions as part of our effort to isolate endogenous microsomal factors that might stabilize glucose 6-phosphatase. We were surprised to find that, on examination of the preparations by SDS/polyacrylamide-gel electrophoresis, only one protein-staining polypeptide was not precipitated by this heat treatment (Fig. 1). This protein fraction was resolved into two polypeptides by gradient gel electrophoresis (see Fig. 4). This doublet exhibited the same mobility during electrophoresis as the low-molecular-weight protein bands (Mr 18 500 and 17 000) in fraction 6 of the conventional purification (see Fig. 1). The heat-stable proteins, although apparently not sufficiently denatured to precipitate, did not exhibit glucose 6-phosphatase activity. However, we were able to prepare 2.5–6 mg of heat-stable proteins from 150 g samples of rabbit liver for use as antigens.

Specific immunoprecipitation of glucose 6-phosphatase activity from solubilized rabbit liver microsomal fractions

Our strategy to raise antisera against the five major proteins in fraction 6 of the conventional purification procedure was somewhat simplified for the low-molecular-weight protein bands. Antisera raised against these heat-stable proteins were used in an attempt to immunoprecipitate glucose 6-phosphatase activity from cholate supernatant (fraction 1) (see the Materials and methods section). Either a fixed amount of fraction 1 (80 μg of protein) and an increasing amount of antiserum was used in the incubation mixtures (Fig. 2b) or a fixed quantity of antiserum (2.5 mg of protein) and an increasing amount of fraction 1 was used in the incubation mixtures (Fig. 2a). Both titration experiments produced similar results, which demonstrated that up to 87% of the glucose 6-phosphatase activity can be specifically immunoprecipitated in the presence of 0.5% (w/v) cholate. The enzyme activity was recovered in the immunoprecipitate pellet, even after the pellets had been washed five times with buffer A, which contains 1% (w/v) cholate. Pre-immune serum precipitated only up to 5% of the glucose 6-phosphatase activity when used in similar titration experiments. Table 2 shows that, when mixtures of
antiserum and pre-immune serum were used together in the same incubation mixture, immunoprecipitation of glucose 6-phosphatase activity is entirely dependent on the volume of antiserum present. Microsomal UDP-glucuronosyltransferase and glutathione S-transferase activities were also measured after addition of phosphatidylcholine liposomes by using sensitive assays. Both of these enzyme activities remained in the soluble supernatants. Thus glucose 6-phosphatase was apparently specifically immunoprecipitated by anti-(heat-stable protein) serum.

The specificity of the immunoprecipitation was confirmed by SDS/polyacrylamide-gel-electrophoretic analysis (Fig. 3). The immunoprecipitates obtained with antiserum (Fig. 3, tracks d and g) contain only two extra polypeptide-staining bands more than the precipitates obtained by using preimmune serum (Fig. 3, tracks b, c and f); these two extra polypeptides exhibit the same electrophoretic mobility as the heat-stable proteins (Fig. 3, track h). Fig. 3 (track e) shows the polypeptides present in the immunoprecipitate obtained by reaction between equal volumes of fraction 1 and antiserum, when approx. 10% of the glucose 6-phosphatase activity was recovered in the pre-

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Fig. 2. Immunoprecipitation of glucose 6-phosphatase activity from solubilized rabbit liver microsomal fraction

Antiserum raised against heat-stable protein was used to precipitate glucose 6-phosphatase activity from solubilized microsomal fraction in the presence of 0.5% cholate (see the Materials and methods section). ⚫ Antiserum; ○ pre-immune serum. (a) Percentage of glucose 6-phosphatase activity found in the immunoprecipitate with a fixed quantity of serum (2.5 mg of protein) and increasing amounts of fraction 1. (b) Percentage of glucose 6-phosphatase activity found in the immunoprecipitate with a fixed amount of fraction 1 (80 μg of protein) and increasing amounts of serum. The mean values of at least three separate determinations are shown, and the ranges are indicated by bars.

Table 2. Immunoprecipitation of glucose 6-phosphatase activity from solubilized microsomal fraction with anti-(heat-stable protein) sera

Results are expressed as the percentage of enzyme activity present in the incubation mixture before centrifugation and are the mean values of at least three separate experiments. Numbers in parentheses shown the ranges of values obtained.

<table>
<thead>
<tr>
<th>Sera in incubation mixture</th>
<th>Glucose 6-phosphatase activity (%)</th>
<th>Resuspended immunoprecipitate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-immune serum (50 μl)</td>
<td>97 (95–100)</td>
<td>3 (0–5)</td>
</tr>
<tr>
<td>Pre-immune serum (25 μl) + antiserum (25 μl)</td>
<td>63 (58–69)</td>
<td>37 (31–42)</td>
</tr>
<tr>
<td>Antiserum (50 μl)</td>
<td>28 (19–37)</td>
<td>72 (63–81)</td>
</tr>
<tr>
<td>Pre-immune IgG (90 μl)</td>
<td>68 (44–98)</td>
<td>29 (0–52)</td>
</tr>
<tr>
<td>Anti-IgG (90 μl)</td>
<td>29 (17–40)</td>
<td>46 (36–60)</td>
</tr>
</tbody>
</table>

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Specific immunoprecipitation of glucose 6-phosphatase activity by using purified IgG fraction

IgG fraction was purified from anti-(heat-stable protein) serum and preimmune serum. These IgG fractions were then made to react with samples of cholate supernatant (see the Materials and methods section). The immune reaction was not very good in the presence of 0.5% (w/v) cholate, and the cholate concentration had to be lowered to 0.1% (w/v) to obtain immunoprecipitates (see the Discussion section). Unfortunately, at 0.1% (w/v) cholate some proteins in the cholate supernatant are very poorly soluble. Thus non-specific precipitation of glucose 6-phosphatase activity can occur. Non-specific immunoprecipitation of glucose 6-phosphatase was eliminated by vigorous washing with buffer A containing 1% (w/v) cholate. Table 2 shows that glucose 6-phosphatase activity was selectively removed from solution by anti-(heat-stable protein)

Fig. 3. SDS/polyacrylamide-gel electrophoresis of immunoprecipitates obtained from solubilized rabbit liver microsomal fraction

Slab electrophoresis was performed in a 4–23% linear gradient of separating gel (18 cm × 8 cm) and a 3% gel stack in the presence of 0.1% SDS (see the Materials and methods section). Tracks: (a), precipitate obtained by incubating cholate buffer with hen antiserum; (b) (c) and (f), precipitates obtained by using preimmune serum; (d) and (g), immunoprecipitates obtained by using antiserum; (e), immunoprecipitate obtained by using antiserum when only approx. 10% of the glucose 6-phosphatase was precipitated; (h), purified heat-stable proteins. Abbreviations: H, IgG heavy chain; L, IgG light chain. Details of the staining procedure are in the legend to Fig. 1.

cipitate and only small amounts of the two extra polypeptides are visible in track (e).

A considerable number of other polypeptides that are visible in the immunoprecipitates can be caused by incubation of equal volumes of cholate buffer with antiserum (Fig. 3, track a) or incubation of fraction 1 with 2.5 mg of serum albumin (gel not shown). These are non-specific effects and do not cause precipitation of glucose 6-phosphatase activity (see the Discussion section). Thus, although a large and inconsistent number of polypeptides can be precipitated during these incubations in the presence of 0.5% cholate, only when antiserum is mixed with fraction 1 are the heat-stable proteins and glucose 6-phosphatase activity specifically and consistently immunoprecipitated from solution.

This work indicates that one or both of the heat-stable proteins would appear to be a glucose 6-phosphatase.

Fig. 4. SDS/polyacrylamide-gel electrophoresis of purified glucose 6-phosphatase

Slab electrophoresis was performed in a 4–23% linear gradient of separating gel (18 cm × 8 cm) and a 3% gel stack in the presence of 0.1% SDS (see the Materials and methods section). Tracks: a (a), precipitate obtained by using pre-immune IgG: a (b), immunoprecipitate obtained by using anti-(heat stable protein) IgG: (a) c and (a) d, purified heat-stable proteins (3 μg of protein); b (a), standards of known molecular weight (2 μg of protein standard), b (b), heat-stable protein plus standard proteins. Abbreviations: OV, ovalbumin; CA, carbonic anhydrase; My, myoglobin; Cyt c, cytochrome c; H, IgG heavy chain; L, IgG light chain. Details of the staining procedure are in the legend to Fig. 1.
IgG. Activity was present in the resuspended immunoprecipitate, although the vigorous washing procedure released some activity into the 'washings' (Table 2).

SDS/polyacrylamide-gel-electrophoretic analysis of the immunoprecipitates was performed to confirm the specificity of the immunoprecipitation (Fig. 4a). The precipitate obtained after incubation with anti-(heat-stable protein) IgG fraction (track b) contained only two extra polypeptides, as well as IgG, more than the few polypeptides in very small precipitate from the pre-immune IgG incubation mixture (track a). Under these conditions the pre-immune IgG is not precipitated and the small pellet obtained after centrifugation contains a few polypeptides also present in similar amounts in the immunoprecipitates. Thus those contaminant polypeptides that are not selectively immunoprecipitated do not relate to glucose 6-phosphatase activity.

The two extra polypeptides selectively precipitated by anti-(heat-stable protein) IgG (Fig. 4a, track b) exhibit the same electrophoretic mobility as the heat-stable proteins (Fig. 4a, tracks c and d). The electrophoretic mobility of the heat-stable proteins when compared with known standards indicates two polypeptides of $M_r$ 18 500 and 17 000 (Fig. 4b).

Discussion

The final specific activity of the rabbit liver phosphohydrolase, purified up to 65-fold from the cholate-soluble fraction by conventional methods, was up to 17 units/mg of protein, which is some 3-fold better than any previously purified preparations of this enzyme.

The enzyme can be stabilized by the presence of 20 mM-NaF in the early stages of solubilization and purification (Burchell & Burchell, 1980), but phospholipids are required to demonstrate activity in the latter stages of purification, as expected from earlier work (Garland et al., 1974; Garland & Cori, 1972).

Our inability to purify the enzyme further by a wide variety of techniques was due to the insolubility of the phosphatase in fraction 6 in a wide range of detergent solutions. This tendency of glucose 6-phosphatase to aggregate and precipitate even in the presence of detergents (Nordlie, 1971) also caused considerable difficulty during immunoprecipitation experiments, especially when using purified immunoglobulin fraction.

The results of our immunochemical experiments in the present paper indicate that an inactive glucose 6-phosphatase protein can be isolated and purified from heat-treated rabbit liver microsomal fractions. We have encountered numerous problems through using antisera raised in chickens. Studies on the characteristics of the antibody response to antigen in chickens (see Benedict, 1967) indicate that maximum amounts of predominantly IgG appear in serum 4 or 5 days after injection, and these rapidly decay during the following days. Thus our immunization procedure may not be inducing the best quality of antisera. Further, the nature of immune-complex formation with chicken antibody is complicated, and often normal serum components are included in the immune complex and serum macroglobulin may co-precipitate when aged antisera are used (see Benedict, 1967). These observations may account for the non-specific precipitation of several proteins in the experiments reported in the present paper, but non-specific precipitation can be eliminated by use of the correct controls (see Fig. 3).

We also encountered problems with the purification of chicken immunoglobulins, as previously recorded (Benedict, 1967). IgG and immunoglobulin M tend to aggregate or polymerize during fractionation in high salt and by chromatography; denaturation leads to precipitation during subsequent dialysis. Thus only a poor separation and purification of IgG from serum proteins was achieved. Our purified antibody exhibited a lower avidity for its antigen.

This 'weak' antibody purified from chicken antiserum does not easily react with glucose 6-phosphatase in the presence of 0.5% (w/v) cholate. However, the immunoprecipitation will occur in 0.1% (w/v) cholate solutions, but extreme washing procedures (see Table 2) must be used to discriminate between immunoprecipitation and non-specific precipitation. In an attempt to improve immunoprecipitation of glucose 6-phosphatase we have tried addition of rabbit anti-chicken IgG as second antibodies, but the addition of these commercially prepared second antibodies did not improve out immunoprecipitation procedure.

Antisera raised against the heat-stable proteins do not inhibit glucose 6-phosphatase activity. The only other antisera raised against glucose 6-phosphatase (anti-microsomal sera) previously obtained appear to inhibit the rat liver enzyme (Schulze & Speth, 1980). Presumably these different antisera interact with different portions of the glucose 6-phosphohydrolase proteins or different components of the glucose 6-phosphatase system; the anti-microsomal preparations could interact with the putative translocase (Ballas & Arion, 1977) for glucose 6-phosphate or mask the active site on the phosphohydrolase.

Anchors & Karlovsky (1975) have successfully purified a cerebral glucose 6-phosphatase to high specific activity (7.8 units/mg of protein). The brain glucose 6-phosphatase ($M_r$ 28 000) appears to be larger than the protein isolated from hepatic endoplasmic reticulum.

A previous paper on the subfractionation of
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Kaderbhai & Freedman, 1980) demonstrated that fractions enriched in glucose 6-phosphatase activity could be produced. The authors were obviously reluctant to relate the enrichment of glucose 6-phosphatase activity to a particular polypeptide-staining band on an SDS/polyacrylamide gel. However, the fraction exhibiting the highest specific activity towards glucose 6-phosphate contained an enrichment of a polypeptide of Mr 18 500. We have confirmed this centrifugal subfractionation work and we are able to obtain a 10-fold enrichment of glucose 6-phosphatase activity corresponding to the appearance of an Mr-18 500 polypeptide (A. Burchell, unpublished work).

Thus evidence obtained by conventional purification procedures, immunoprecipitation experiments and more recently by centrifugal analysis indicates that glucose 6-phosphohydrolase is a low-molecular-weight polypeptide, possibly of Mr 18 500.

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References
